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Self-Incompatibility Systems as Bioassays for Mutagens

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Many flowering plants are unable to set seeds with their own pollen because a system known as gametophytic self-incompatibility is operating. The basis of this system is a single multiallelic locus S, and if the S allele carried by a pollen grain matches one of the two S alleles carried in the style, as it is certain to do upon self-pollination, then pollen tube growth is inhibited. Should one of the self-pollen grains carry a mutated S allele, however, it would not match either of those carried in the style and would therefore, not be inhibited. Gametophytic self-incompatibility thus provides a mechanism for discriminating between such mutant and nonmutant pollen grains. Knowing the numbers of pollen grains available to the stigma, and also the numbers of seeds produced, it becomes possible to estimate the frequency with which mutations occur at the S locus. Assay systems of mutagenesis which employ gametophytic self incompatibility will allow very large numbers of pollen grains to be screened for S allele mutants, which should indicate the mutagenicity of the environment. These systems have the added benefit that screening is done by the stylar tissues, rather than technicians. Finally, they may be used to construct largely autonomous assay systems which would provide continuous monitoring of the environment.

Flowering plants have evolved a variety of mechanisms which insure that accidental self-pollination is not followed by self-fertilization. Such mechanisms are known as self-incompatibility systems and generally function by preventing either the germination or the tube growth of self pollen. Gametophytic forms of self-incompatibility, certainly the most widespread type in existence, are so named because their function basis is the haploid, or gametophytic, genotype that is carried within the individual pollen grains.

In the simplest case of gametophytic self-incompatibility, a single multiallelic locus, S, controls the reaction between pollen and style. If the S allele in the pollen matches either of the two S alleles in the stylar cells, pollen tube growth is inhibited. It is important to note that, with this form of self-incompatibility, pollen grains from a single plant differ in their incompatibility type; this fact provides the basis of a system for monitoring environmental mutagenesis. If a plant possesses a rigorous form of gametophytic self-incompatibility, numerous self-pollinations, involving many millions of pollen grains, will not result in a single seed, unless, however, the S allele carried by a particular pollen grain has mutated. In that case, the pollen S allele will not match those of the style, and pollen tube growth will proceed normally, resulting in fertilization and the formation of a seed. Determining the numbers of seeds resulting from pollination by a known number of pollen grains provides a good measure of mutation rates at the S locus.

Advantages of the S System as a Bioassay for Mutagenesis

Devreux and de Nettancourt (*1*) have pointed to two major advantages demonstrated by the self incompatibility system. (1) Since pollen is a haploid system, the difficulties of detecting recessive alleles do not arise as they do in diploid systems. Thus, most mutations in the S allele will be expressed. (2) If mutant pollen tubes are allowed to effect fertilization, the mutant genotype can be recovered and subjected to further study and verification. This is particularly important since, to this point, no S allele has ever been changed by a mutagenic agent from one specificity to another. That is, S_1 mutates to S_2. Instead, all mutations have represented deactivation of some component in the S allele, thus S_1 mutates to S_1'. This deactivation may be limited to the pollen, to the style, or exhibited by both (*2*). Detection of a new specificity among presumptive

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mutants would thus certainly indicate contamination rather than mutation.

To these important advantages, we may add the following. Very large numbers of pollen grains can be screened with little effort. The stigma of *Oenothera organensis* will accommodate nearly 5,000 pollen grains, a fact which allowed Lewis (*3*) to screen 65×10^6 pollen grains for mutants in the S allele. Taking advantage of the small pollen grains and large stigmatic surfaces of *Nicotiana alata*, Pandey (*4*) found that over 17,000 pollen grains could be screened in each pollination. These large numbers allow rare mutational events to be detected and their frequencies to be measured with great accuracy.

Another major advantage of the self-incompatibility system is that the actual screening of mutant versus nonmutant pollen grains is accomplished by the stylar tissues themselves. This eliminates the need for great skills on the part of technicians.

Environmental mutagenesis is very likely to vary greatly over both time and space. Thus, it becomes imperative that *in situ* assay systems be developed. Several of the species carrying the S allele may provide this capability and, as outlined in a following section, it now seems likely that a largely autonomous assay system, employing the self-incompatibility system, may be feasible. This would allow a significant increase in our capacity to measure environmental mutagenesis.

Disadvantages of the S System

As is discussed below, previous studies of the self-incompatibility systems have indicated that they are presently less sensitive to mutagenesis than is the *Tradescantia* stamen hair system, for example. Until now, however, no efforts have been made to maximize sensitivity of the system and many opportunities exist for doing so. It thus seems likely that this disadvantage can be overcome.

Although the self-incompatibility system is often extremely effective in discriminating between self and "other" pollen, this is not so in some species. *Cheiranthus cheiri* (Cruciferae) exhibits what is termed, "cryptic self incompatibility." That is, it rigorously excludes self pollen tubes when pollinated with a mixture of self and other pollen. However, it is fully self fertile when pollinated with only its own pollen (*5*). In other cases, a wide spectrum of environmental stresses as well as aging and, possibly, infection, may significantly weaken the self-incompatibility system (*6*). Susceptibility to such influences is apparently a clonal characteristic (*7*) and thus careful selection of plant material will greatly diminish this possible source of error (*2*).

Nature of S Allele Mutations

Two general models of S allele mutations have been suggested. The first of these is based on Lewis's (*3, 8*) now widely accepted suggestion that the S allele is actually tripartite, one component of which codes for the S allele specificity (S_1 or S_2, etc.), another which activates the incompatibility system in the style, and a third for activation in the pollen. Lewis (*3, 8*) was the first to suggest that simple point mutations could destroy the specificity code or the pollen activator of self incompatibility, either of which would result in a failure to reject "self" pollen. Plants bearing such mutations are thus capable of self pollinations.

A second model for mutations to self compatibility was proposed by Brewbaker and Natarajan (*9*). It is based on the finding by Lewis (*10*) that in some systems of gametophytic self-incompatibility, the presence of two, rather than one S allele, may severely limit or even eliminate the self incompatibility reaction. The presumed explanation for this fact is that competitive interactions between the two S alleles prevent either one from functioning normally. Brewbaker and Natarajan (*9*) employed this hypothesis to explain why, after irradiating plants of *Petunia* to induce self-compatibility, they found that each resultant mutant carried an extra centromere-bearing fragment of chromosome. They postulated that these fragments carried an S allele which interfered with the functioning of the normal S allele, thus inducing the self-fertile condition. Another view is that these fragments may serve also (or instead) to complement a genome which contains an otherwise lethal mutation and it is this mutation which causes the self-compatibility (*4*). More recently, de Nettancourt et al. (*11*) presented the very interesting hypothesis that centric fragments in *Nicotiana alata* may contain a nucleolar organizing region and, while the normal n.o.r. of the pollen genome is blocked by the self-incompatibility reaction, the n.o.r. of the fragment functions, thereby circumventing inhibition of pollen tube growth. In other studies, however, 11 out of 24 self-compatible mutations in *Nicotiana alata* were found to be free of centric fragments (*12*), although Pandey (*4*) has argued that chromosomal fragments may be inserted into the normal karyotype and carried there rather than on centric fragments. This may indeed be the case, but since centric fragments have never been associated with induced self compatibility in any species outside the Solanaceae (*12*), and Lewis (*13*) has presented evidence that they do not occur in *Oenothera organensis* (Onagraceae), we must conclude that they are not inevitable associates of S allele mutations.

The possibility that extra chromosomal segments

may be required to induce self compatibility in some taxa is an important consideration because such fragments will not result from a single chromosomal break. Instead they require two breaks and, because the probability of accomplishing this is reduced, low levels of radiation or low concentrations of mutagens will not be very effective in inducing multiple breaks. Indeed, van Gastel and de Nettancourt (*14*) found that chronic γ-irradiation was almost totally ineffective in the induction of mutations to self compatibility. They suggested however, that this may be due to the fact that only very small doses of irradiation could be absorbed during the brief period of maximum sensitivity, the meiotic metaphase I. Thus, although the total dose accumulated may be large, most of this occurred during periods of low sensitivity to mutagenesis.

Sensitivity of the Self-Incompatibility System to Induced Mutation

Certainly one of the most successful higher plant assays for mutagenesis is that of determining the frequency with which stamen hairs of *Tradescantia* change from blue to pink (*15*). Accordingly, it may be useful to compare the sensitivity of the *Tradescantia* stamen hair system with the self-incompatibility system. Figure 1 shows a dose response curve for *Tradescantia* clone 02 (*15*). The mutagenic agent was x-ray, and the frequency of mutations to pink cells are indicated. The spontaneous mutation rate (control) has been subtracted from the values shown. Also shown in Figure 1 are data obtained by Lewis (*3*) with self-incompatibility systems of *Prunus avium* and of *Oenothera organensis*. For these studies, the mutagenic agent was also x-ray, although given in roentgens. The spontaneous mutation rate has been subtracted from the values shown. (To allow comparisons between rads and roentgens of x-rays, roentgens should be multiplied by 0.96, although the gross differences between these studies largely obviate the utility of this correction.)

Among several studies of mutability at the S locus, that on *Prunus avium* provides the most nearly complete dose response curve, and clearly, there is a significant difference between the *Tradescantia* stamen hair curve and the *Prunus* self incompatibility curve. Several factors may function to produce this difference.

Sparrow et al. (*16*) have demonstrated that radiosensitivity may be considered in terms of target theory, and the genetic basis for pigment production in *Tradescantia* stamen hairs may perhaps present a

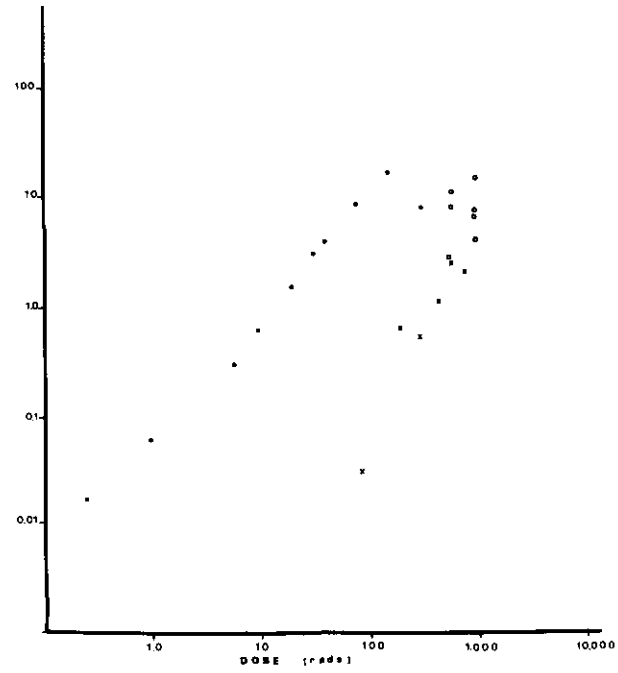

FIGURE 1. Dose-response curves for *Tradescantia* 02 clone mutations to pink, and for *Prunus avium* and *Oenothera organensis* mutations to self compatibility. (●) *Tradescantia* pink mutations/100 hairs clone 02 (minus control); (x) *Prunus avium*, fruits/100/flowers (minus control); (○) *Oenothera organensis*, pollen tubes/100 flowers (minus control). Data after Nauman et al. (*15*) and Lewis (*3*).

larger total target area than does the S locus of *Prunus* or *Oenothera*. In that case, the stamen hair system would be inherently more sensitive than is the self incompatibility system.

Another factor that must be considered is the difference between numbers of pollen grains and numbers of viable pollen grains. Mutagenic agents invariably reduce the porportion of viable pollen grains contained in any treated pollen sample. Thus, the number of viable grains used in pollinations decreases, often quite drastically, with higher doses of mutagen. Figure 2 was originally published by Evans (*17*) and shows the decline in pollen viability which accompanies increasing exposure to acute x-ray irradiation. In other studies, the decline has not been so dramatic: van Gastel and de Nettancourt (*12*) found that 300, 600, and 825 rads of x-rays reduced pollen viability in *Nicotiana alata*, as indicated by staining with iodine, to 81.9%, 63.2% and 51.6% respectively. Exposure to 825 rads of x-rays reduced the germinability of *Tradescantia paludosa*, in comparison, to approximately 5.0% (*17*). Part of this difference between the two studies may be due to differential radiosensitivity (*18*). Another important factor is that germinability (used

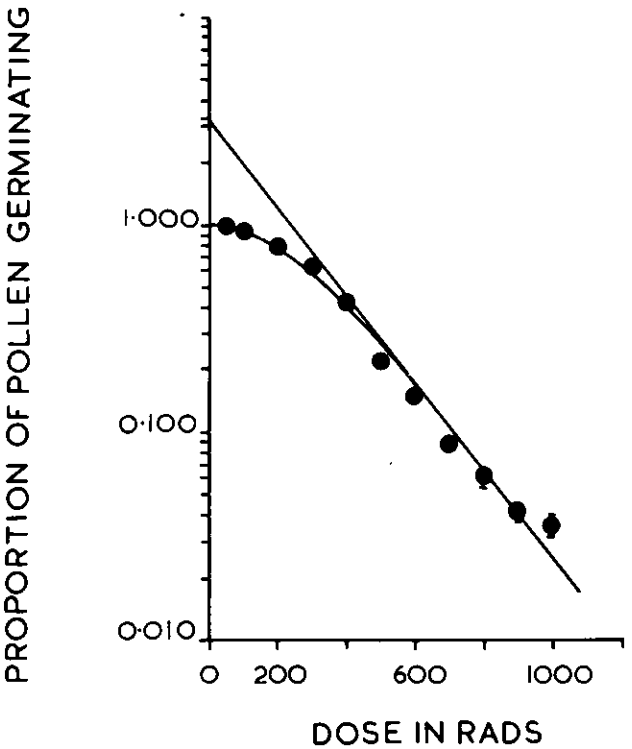

FIGURE 2. Survival curve for pollen grains of *Tradescantia paludosa* irradiated with x-rays 8 days prior to dehiscence: D_0 = 204.9 ± 11.2 rads n= 3.34 ± 0.43. Data of Evans (*17*).

as a criterion by Evans), is frequently a much more accurate indicator of viability than is stainability, used by van Gastel and de Nettancourt. Exceptions to this differential are those cases where staining requires active metabolism. Fluorescein diacetate is one such exception (*19, 20*). It is thus very likely that doses of 600–1000 rads, frequently employed by investigators of the incompatibility system, may be reducing pollen viability to a small fraction of control values and many of the pollen grains placed on the stigma are incapable of germination. Although some investigators have allowed for this factor (*12, 14*), the frequency of S allele mutants may sometimes be grossly underestimated. Consequently, it is not presently possible to determine how sensitive the self incompatibility system is as an assay for mutagenesis. With few exceptions, for example, van Gastel and de Nettancourt (*12, 14*) investigations of mutations to self-compatibility have been concerned with the nature, rather than the quantity, of mutational events. Thus, no attempts have been made to develop systems which will be sensitive to mutagenesis. The following section presents evidence which suggests that such attempts would be very fruitful.

Future Development of Self-Incompatibility Systems as Assays for Environmental Mutagenesis

To maximize the utility of self-incompatibility systems as assays for mutagenesis, the following tasks must be accomplished: (1) species which exhibit a high frequency of induced self compatibility mutations should be identified; (2 within these species, clones which exhibit a high degree of self incompatibility should be selected; (3) techniques for employing the selected clones as *in situ* assay systems should be developed.

The first goal, that of identifying species which exhibit a high frequency of induced mutations has perhaps been brought within reach by investigations of complex systems of self incompatibility. Lundquist (*21, 22*) has shown that in several species of grasses (*Secale cereale, Festuca pratense, Hordeum bulbosum,* etc.) self incompatibility is gametophytically controlled and involves two distinct loci, termed S and Z. Inhibition of pollen tube growth requires that alleles found at both of these loci in the pollen must be matched by alleles carried in the style. A pollen grain of the genotype S_1Z_3 would be inhibited in a style of the genotype S_1S_2 Z_1Z_3. S_1Z_3 pollen, would not, however, be inhibited in an S_2S_3 Z_1Z_3 style. Thus, if either the S or the Z allele in the pollen is inactivated by mutation, pollen tube growth proceeds without inhibition. Presuming that the mutation rates at the S and Z loci are comparable to those of other taxa, we should thus expect the frequency with which pollen grains carry a mutation to self-compatibility in the grasses should be twice the frequency of that in taxa which carry only one self-incompatible allele. Furthermore, *Ranunculus acris* pollen grains each carry three loci (S_a, S_b, S_c), all of which must be matched in the style to cause inhibition of pollen tubes (*23*) and in *Beta vulgaris* pollen must be matched in no fewer than four loci (S_a, S_b, S_c, S_d) (*24*). These last two taxa should thus exhibit three and four times the probability of mutating to self compatibility than do single locus taxa. Presently, as was the case with most other examples of self incompatibility (*6*), it is necessary to identify clones of both *Ranunculus acris* and *Beta vulgaris* which exhibit a rigorous degree of self-incompatibility. The product of such selections may be new and highly sensitive assays for mutagenesis.

In addition to increasing the sensitivity of the incompatibility system to mutation and also selecting for increased rigor in the rejection of self pollen, the development of largely autonomous, *in situ* assay

systems would be of great utility.

Requirements for such a system are surprisingly modest and met by many self incompatible species, including all of the above mentioned multilocus systems (the Gramineae, *Ranunculus acris*, and *Beta vulgaris*). The species should be capable of vegetative reproduction, in order that useful clones can be produced and maintained. Fruits produced by the flowers should be uniovulate so that it is not necessary to induce fruit development artificially. In each of the currently operative self-incompatibility assay systems (*Oenothera*, *Nicotiana*, and *Petunia*) the number of seeds borne in the ovary is quite large. This quality is generally considered advantageous in that it allows recovery of several mutants from one pollination. With it, however, comes the requirement that each ovary be treated with lanolin containing β-naphthoxyacetic acid in order to prevent abscission of fruits which contain only a fraction of the normal complement of developing seeds.

Members of the Gramineae, *Ranunculus acris*, and *Beta vulgaris* all produce achenes; that is, one-seeded fruit. This eliminates the necessity of inducing parthenocarpy. A final requirement for the proposed autonomous assay is that the self incompatibility system of the test species may be circumvented in order to allow self-fertilization during establishment of the test material. This, too, is easily accomplished, since bud pollination is known to be frequently effective in this respect (*25*).

To construct an autonomous assay system in a species showing the prerequisites of sensitivity, rigor, and vegetative propagation, it is necessary to first self-fertilize one clone. If the original clone was heterozygous at one locus, e.g., S_1S_2, the resultant progeny will contain the genotypes S_1S_1, S_1S_2, and S_2S_2. Of these, the heterozygote S_1S_2 will be able to fertilize both its S_1S_1 and S_2S_2 siblings. The homozygotes will be interfertile but neither one will be able to fertilize the heterozygotes. If rametes of an S_1S_1 clone are interplanted with those of an S_1S_2 clone, a very interesting situation is created (see Fig. 3). A flower of gentotype S_1S_1 will receive some pollen which is produced by itself or by other flowers on the same plant, but I shall ignore this quantity, in part because in a large population it is probably very small in comparison to the amount of pollen received from other plants, and in part because only specific field tests will allow any estimation of what this quantity is. That same S_1S_1 flower will receive pollen also from other plants of genotype S_1S_1, all of which will normally be rejected as incompatible. Furthermore, an equal quantity of pollen will be received from plants of genotype S_1S_2, half of which will be rejected and half of which will not. Thus, one-quarter of pollen received by an S_1S_1 flower is compatible (S_2) and three-quarters is incompatible (S_1). For every 1000 seeds produced on clone S_1S_1 we may assume that 3000 seeds were prevented from forming by the incompatibility system. Rametes of clone S_1S_2 should produce no seeds, but, for every 1000 seeds borne on clone S_1S_1, clone S_1S_2 should have received a quantity of pollen which, if compatible, would have resulted in the production of 4000 seeds.

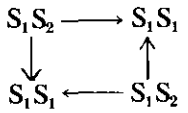

FIGURE 3. An autonomous, *in situ*, assay for environmental mutagenesis. S_1S_2 indicates a plant which is self incompatible and heterozygous at the S locus. One half of its pollen, that carrying the S_2 allele, will be compatible on S_1S_1 stigmata. This compatible pollen flow is represented here by arrows and it will serve as a control, indicating how many seeds would be produced when compatible pollen is available. For S_1S_2 plants, the only compatible pollen grains will be those which carry a mutated S allele. Seed production by S_1S_2 plants, as a fraction of that in S_1S_1 plants, will thus measure the frequency of mutation.

Any seeds produced on clone S_1S_2 will presumably carry mutants in the S locus. Harvest and planting of these seeds will allow rigorous testing of this presumption. The mutation rate may then be stated in terms of x mutants retrieved from clone S_1S_2 per 1000 seeds produced in clone S_1S_1. This has the great advantage of allowing for changing quantity and quality of pollen and ovules produced by the plants throughout the flowering season. The result would be a true *in situ* assay system, monitoring the environment for long periods of time and perhaps detecting transient but biologically significant mutagenic events. The numbers of pollinations made would be many times those which could be made by experimentalists. Furthermore, such a system could be composed of individuals which carry easily identifiable electrophoretic markers. This would enable determining if presumptive mutants were derived from a single pollen source and thus perhaps represented a large mutant sector within one plant or if many plants contributed mutated pollen, indicating a high frequency of mutation.

On the basis of these and other considerations, such a system would enable self-incompatibility to provide useful monitors of environmental mutagenesis.

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